Universidade de Aveiro Departamento de Biologia
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Filipa Alexandra Lourenço Baptista

AVALIAÇÃO DA APLICAÇÃO DE EQUIPAMENTO ELECTRÓNICO NA CONTAGEM E DIFERENCIAÇÃO CROMÁTICA EM ENSAIOS DE ECOTOXICOLOGIA

EVALUATION OF THE APPLICABILITY OF AN ELECTRONIC DEVICE IN COUNTING AND CHROMATIC DIFFERENTIATION IN ECOTOXICOLOGY ASSAYS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica de Doutor Sizenando Nogueira de Abreu do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar, e Doutor Miguel Augusto Mendes Oliveira e Silva do Departamento de Engenharia, Eletrónica, Telecomunicações e Informática da Universidade de Aveiro.

O Júri

Presidente

Prof. Doutor João António de Almeida Serôdio
Prof. Auxiliar com Agregação, Departamento de Biologia da Universidade de Aveiro

Doutor Fernando Manuel Raposo Morgado (arguente)
Prof. Associado com Agregação, Dep. Biologia, UA

Doutor Sizenando Nogueira de Abreu (orientador)
estagiário Pós-Doutoramento, CESAM UA

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palavras-chave

D counter, Contagem e diferenciação cromática, Contagem automática de organismos, Ensaios biológicos em Ecotoxicologia, *Daphnia magna*, Dureza, Amónia

resumo

O presente estudo foi realizado no âmbito da Biologia Aplicada tendo como objetivo geral a avaliação da aplicabilidade de um novo dispositivo (D counter) em ensaios biológicos no âmbito da Ecotoxicologia.

O trabalho desenvolvido apresentou três fases distintas: uma fase inicial com adaptação aos comandos e funcionalidades do novo equipamento, uma segunda fase com aplicação da metodologia clássica em paralelo com a abordagem otimizada pelo equipamento D counter, e uma terceira fase com a execução de um teste ecotoxicológico, usando unicamente as potencialidades oferecidas pelo D counter, focando-se principalmente na contagem de caracterização cromática de organismos.

Avaliações preliminares acerca da aplicabilidade do D counter em contar e diferenciar cromaticamente microrganismos, em testes ecotoxicológicos, foram apresentadas na SETAC2017 (Bruxelas). O resumo estendido encontra-se no capitulo II, onde estão descritas as principais funcionalidades do D counter, cujo objetivo se foca na simplificação e automatização dos testes ecotoxicológicos, por forma a minimizar problemas inerentes ao método clássico de contagem e caracterização de organismos.

Grande parte dos testes com *Daphnia magna* requerem a contagem e medição de elevado número de organismos, o que pode causar erros humanos e prejudicar a acuidade visual do técnico. Desta forma o procedimento de trabalho com o D counter acrescenta objetividade e precisão, economizando tempo e diminuindo o tempo de exposição ao químico testado.

A segunda fase do estudo pretendeu avaliar o D counter em estudos biológicos clássicos. Para tal, comparou-se a performance deste com a abordagem tradicional, através de um ensaio crónico de 21 dias com *D. magna,* por forma a avaliar parâmetros como a reprodução e o crescimento dos organismos, usando os dois métodos, na ausência de contaminante.

Numa terceira fase aplicou-se a nova metodologia a um teste crónico, onde foram estudados os efeitos combinados da variação da dureza da água e amónia em *D. magna*. Os parâmetros estudados foram a reprodução e o crescimento dos organismos, com base nos resultados obtidos no D counter.

De uma forma global, este estudo demonstrou que o novo equipamento não exibe diferenças significativas em comparação com o método clássico ($p = 0,822$), mostrando-se assim capaz de substituir a metodologia atualmente utilizada. Dados do D counter, mostram que a dureza e a amónia demonstraram ser parâmetros que afetam o tamanho e reprodução dos organismos. Meio com valor mais baixo de dureza resulta em organismos com menor tamanho, enquanto o oposto se observou no meio com dureza mais elevada. Contrariamente ao esperado, concentrações mais altas de cloreto de amónia (12 e 20 mg/l) apresentaram maiores taxas de reprodução em comparação com o controlo e concentração mais baixa de cloreto de amónia (2mg/l).

Este estudo demonstra e valida a aplicação de novas metodologias na área da ecotoxicologia, podendo abrir novas potencialidades e evoluções do equipamento no futuro.

keywords D counter, Counting and chromatic differentiation, Automatic counting of organisms, Biological essays, *Daphnia magna*, Hardness, Ammonia

abstract

The present study was developed in the ambit of Applied Biology aiming the evaluation of an innovative device applicability in Ecotoxicology bioassays. The study was developed in three distinct phases: a first phase with adaptation to the commands and functionalities of the new equipment (D counter); a second phase using both classical methodology and the innovative approach (provided by the new device) in bioassays; and a third phase performing a standard bioassay in Ecotoxicology, only assisted by the functionalities available from the D counter, mainly in organism counting and chromatic characterization.

Preliminary results evaluating the applicability of D counter in counting and chromatic differentiation in Ecotoxicology bioassays were presented at SETAC2017 (Brussels). The study presented to the conference is included in chapter II as an extended abstract, enhancing D counter main features simplifying and automating ecotoxicological tests, minimizing many problems inherent to the classical method of counting and characterizing organisms. Many *Daphnia magna* testing requires the counting and measurement of large amounts of organisms, which can lead to human error and even impair the visual acuity of the technician. The working procedure with D counter adds objectiveness and accuracy, saves time, and also reduces the exposure period to the chemical tested.

A second phase of the present study was to evaluate D counter in classical bioassays tests, comparing D counter performance and the traditional approach, through an essay with *D. magna*, considering a chronic test of 21 days, and evaluating parameters such as reproduction and growth of the organisms using both procedures in the absence of contamination.

In a third phase, the new methodology was applied to chronic test, where the combined effects of the variation of water hardness and ammonia in *D. magna* were studied. The studied parameters were reproduction and growth of the organisms exposed to combined effects, considering data only acquired and provided by the new device D counter.

Overall, this study demonstrated that the new equipment does not exhibit significant differences, when compared to the classical method ($p = 0,822$), thus being able to assist or even replace the current counting methodology. D counter data showed that water hardness and ammonia have shown to be parameters that affect the size and reproduction of organisms. Medium with lower hardness results in organisms with smaller size, while the opposite was observed in the medium with higher water hardness. Unexpectedly, higher concentrations of ammonium chloride (12 and 20 mg/l) presented higher reproduction rates, compared to the control and lower concentration of ammonium chloride (2mg/l).

This study presents and validates the application of new methodologies in ecotoxicology, being able to open new potentialities and evolutions of the equipment in the future.

Summary

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Acronym list

ASTM: American Society for Testing and Materials

AFR: age at first reproduction

BL: body length

DIN: German Institute for Standardization

ISO: International Organization for Standardization

SFR: size at first reproduction

OECD: Organization for Economic Co-operation and Development

LC50: median lethal concentration (concentration of a chemical that will cause death to 50% of the organisms exposed to it)

EC50: Half maximal effective concentration (represents the concentration for witch, 50% of the test organisms exhibit a response)

SETAC: Society of Environmental Toxicology and Chemistry

“A ciência serve para nos dar uma ideia de quão extensa é a nossa ignorância”
Félicité Robert de Lamennais

Chapter 1

General Introduction

1.1 Introduction

This chapter provides a general overview of the subject of study, summarizing main concepts evaluated in Ecotoxicology, and pointing main difficulties quantifying several classical endpoints, leading to the urge of innovative technology to be applied in the classical bioassays.

That innovative technology has to be evaluated and applied to classical approach, before its application becomes generalized. Main advantages have to be enhanced but potential limitations have also to be enumerated.

The main objective of this study is to evaluate and validate the applicability of a new equipment (D counter) for counting and discrimination of microorganisms frequently used in ecotoxicological tests, being chosen the crustacean *Daphnia magna*, one of the most used model organism in bioassays.

1.2 Ecotoxicology

Despite the negative impact caused by human activities began some thousand years ago, mainly due to industrial revolution, public awareness on this matter started in the middle of the 20th century. In fact, only after the publication of Rachel Carson's famous book, "Silent Spring", in 1962, did the public opinion became aware of the negative impact of chemicals on the environment (Vighi & Villa 2013). In Ecotoxicology, the harmful effects of chemical pollution in the environment are studied by means of bioassays tests. In fact, ecotoxicology is an area of science that focus on the study of the effects of anthropogenic chemicals on ecosystems, at different levels of biological organization. Basically, it helds within a sequence of events which primarily consists of identifying contaminants, studying their behaviour and finally assessing their effects on the biosphere. The purpose of this sequence is essential to: determine the level of contaminants; assess the degree of danger of contaminants and their metabolites in living organisms; estimate the maximum permitted levels of contaminants; diagnose the effects on the environment as well as the effects of the measures taken in order to, finally, evaluate the ecological risks (Costa & Olivi 2008).

Ecotoxicological tests, also called bioassays, can be performed with aquatic or terrestrial organisms, depending on the study to be performed, and may cover various levels of organization, from the individual to the ecosystem (Connon at al. 2012). These studies can be short or extended for over several years. Nevertheless, it should be noted the need for a large number of tests with organisms belonging to different trophic levels. These biological assays aim to evaluate of the adverse effects of a toxic substance or a complex mixture for aquatic organisms (Cooney 1995; Walker *et al.* 2001). The response of organisms to toxic contaminant depends on the time of exposure as well as the sensitivity of the species, community or ecosystem to a chemical (Connon at al. 2012).

Biological assays normally require the exposition of the test organisms to different concentrations of the substance of interest (Barroso 2009). A parameter of large importance is the ratio between the amount of chemical, to which the organism is exposed, and the consequent toxic effects. In order to assess the risk that a chemical comprises to the environment, the dose-response parameter must be analysed (Rodrigues 2010). However, ecotoxicological tests have several limitations, namely in terms of interactions and/or transformations of the chemical substances in the aquatic environment (Magalhães & Filho 2008). Despite these obstacles, the ecotoxicological studies are an essential tool for the study of toxicity in aquatic environments because they can expose potential risks of numerous anthropogenic compounds (Martins 2013).

Toxicity tests are standardized by international organizations, such as OECD (Organization for Economic Cooperation and Development), ASTM (American Society for Testing and Materials), ISO (International Organization for Standardization), DIN (German Institute for Standardization), among others. The most common ecotoxicological tests are the acute and chronic tests. Acute tests aim to evaluate mortality or immobility of the test organism when exposed to contaminants and last for a period of less than 96 hours of exposure. The recommended duration according to OECD is 24 or 48h (OECD 2008). The main goal of acute tests is to determine the median lethal concentration (LC50), which is defined as the concentration of the test substance likely to cause 50% mortality of the population of the test organisms (EPA 2002). When aiming for the study of the

toxic effects upon vital and/or functional of the organism, the EC50 index is applied. This index, represents the concentration for witch, 50% of the test organisms exhibit a response (Connon at al. 2012).

Chronic toxicity tests are long term tests designed with the objective of studying non-lethal effects on the organisms from their prolonged exposure to sub-lethal concentrations of a chemical substance. The effects are usually assessed by the specific analyses used for the detection of chronic changes, such as physiological disorders, alterations in the growth and reproduction rates of the organism (EPA 2002)**.** In ecological terms, the chronic tests allow to measure parameters for which the acute tests do not provide information. Thus, from the standpoint of complementary environmental assessment and monitoring, chronic testing becomes more biologically relevant. For example, chronic tests are more sensitive to media dilution, expected in environmental samples (ex: industrial effluent), which evaluate the response of pollutants over a longer period of time (Magalhães & Filho 2008).

Numerous studies require both the counting and chromatic characterization of organisms, and other particles, with reduced dimension, for the evaluation of several parameters. Nowadays, this procedure is made by naked eye, leading to errors bind to the sensibility of the technician in charge, and it may even cause visual acuity problems. Hence, there are a few limitations to these tests that can cause a reflection on the results obtained. Most of these limitations are liked to direct human error due, not only to the high number of organisms within each test, but also due to prolonged exposure to certain chemicals. Therefore, different concentrations of the chemical in a study will require the counting process to be repeated for each concentration times its replica, where the number of organisms being used at each test concentration is reduced from at least 40, preferably divided into four groups of 10 organisms (OECD 2008). These factors can induce both physical and psychological distress, which can result on the perception and collection of biased data.

Thus, the development of an equipment able to automatize certain protocols procedures, would be an asset for the field of ecotoxicology.

1.3 Automatic counting and chromatic differentiation of organisms – D counter

The D counter (figure 1) is an automatic counting and chromatic analyse device able to deal with both microorganisms and particles in suspension in aquatic medium, ex. *Daphnia sp*. as well as others with reduced dimensions. The device is an asset for ecotoxicological tests, due to its ability to automatically count high quantities of microorganisms, measure their body length (BL) and simultaneously the absorbance in the exposure medium.

Figure 1- D counter.

The device presents an interface for computer connection, being the process of control and characterization of particles made by a specific software, developed by its inventors. In each test, the current version of the device forces the passage of every organism serialized through a group of optical sensors. The acquired digital signals (figure 2) are then processed in real-time by a computing system in order to automatically count and characterize each organism. The device can operate either using a module of LASERs or a module of LEDs as light of source to promote the signals. All the acquired data is stored allowing it to be reprocessed using different detection and characterization criteria and also to be exported in common data processing applications (such as Excel).

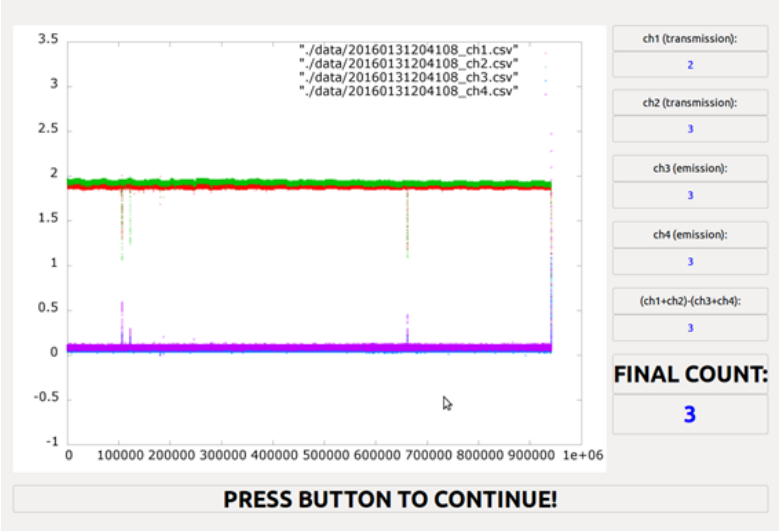

Figure 2- Digital Signal.

This device becomes a promising innovation due to the fact that it allows for a faster and more efficient way to collect data, avoiding most of the previously mentioned sources of error. The thorough counting of the organisms required in the toxicity tests represent an enormous effort from the technicians, mainly when it comes to visual acuity, due to the small size of the specimens. Hence, the time required for this part of the process is long enough to be one of the major causes of the introduction of errors.

The evaluation and validation of D counter was performed using one of the most commonly used test organisms in ecotoxicology tests: *Daphnia magna.*

1.4 *Daphnia magna* as a model species in Ecotoxicology tests

The selection of species is essential in ecotoxicological tests and should respect specific criteria, such as availability and abundance in the environment, ease of cultivation in the laboratory, knowledge of the species biology as well as their characteristics as sensitive organisms (Rand 1995).

The genus *Daphnia*, is considered one of the most common test organisms in biological research, ever since the early 19th century (Ebert 2011; Lampert 2006). Also known as "water fleas", due to their saltatory swimming style, these organisms are small planktonic crustaceans, 0.2-5 mm in length, and became a model in the

ecological studies, due to their great capacity of adaptation to diverse habitats, having a wide distribution around the world like North America, Europe and North Asia (Lampert 2006; Forró et al. 2008).

In fact, their abundant presence in many lakes, makes them a keystone species of sum importance. Moreover, the large number of species from this genus, elevates them to the status of useful tool on ecological and evolutionary studies, due to their ability to reflect adaptations to different habitats (Lampert 1991).

Hence, their ecological relevance as primary grazers of phytoplankton and primary foragers for planktivorous fish, puts them in a central position of the food chain, increasing their importance in studies on interactions between food chains. For this study, it was accessed the vulnerability of *Daphnia* to Ca demand. Therefore, the genus *Daphnia* is directly influenced by the water chemistry and, particularly, by the Ca fluctuations (Alstad et al. 1999), enhancing *Daphnia* as an ideal organism for the evaluation of water chemical parameters.

In addition, the planktonic crustaceans have other important characteristics, which make them a model organism (Jesus 2012; Lampert 2006):

- They have a small size, but large enough to be handled independently;
- Easy to handle and maintain in laboratory conditions;
- They have short life-cycle, and fast reproduction (In less than 10 days, the organisms give offspring, and every 3 days they give a new litter, which under good conditions can reach 50 new-borns);
- Their response to environmental changes is fast;
- Its reproduction is basically made by parthenogenesis, which allows to obtain organisms genetically equal, and their clones can be maintained in laboratory for long time; but they are capable of sexual reproduction, in some specific conditions;
- The body of these organisms is quite transparent, making it possible to visualize the interior; this characteristic is indicated for morphological and physiological studies;

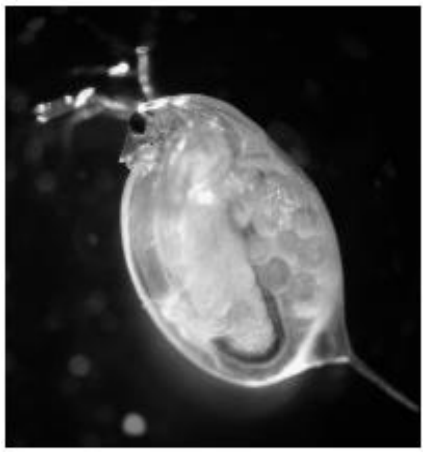

Figure 3- *Daphnia magna* Straus (1820) (adult organism).

The exposition to chemicals is also a relevant factor that should be taken in account. Therefore, the D counter, an automatized device, appears to offer a more efficient and time saving alternative for the counting and characterization of microorganisms which can also estimate *Daphnia* body length (BL).

To all that was mentioned above, it is also important to add the relevance of the use of *D. magna* (figure 3) due to the fact that, the present work, focuses mainly on the use of an innovative new tool for the counting and characterization of microorganisms used in ecotoxicology.

Commonly, chronic tests with *D. magna* last for 21 days require observation of the test organisms every other day, registry of the number of offspring produced and the final size of the test organisms (OECD 2012); thus, allowing the determination of the effects of the chemical substance on reproduction and growth of *D. magna*. The observation of the test organisms is currently being made against a bright light, to perform the manual counting due to their reduced size. The observation under these light conditions facilitates the observation of the organisms, their molts and counting the offspring. Hence, this process is not only time consuming, but also potentially related to human error and even to health risk in the visual care of the laboratory technician in charge, who also have to deal with prolonged exposure to chemicals present in medium.

1.5 Thesis overview

As stated before, the first chapter provides a general overview of the subject of this thesis, summarizing some concepts evaluated in Ecotoxicology, and pointing main difficulties quantifying several endpoints, such as counting or grow inferred from body length (BL) along exposure period, leading to the urge of innovative technology to overcome and simplify the classical approach.

The second chapter, entitled "**D counter: Automatic Organism Counting and Characterization in Ecotoxicology assays**", includes an extended abstract presented in the Society of Environmental Toxicology and Chemistry (SETAC), Brussels, in 2017, where the innovative technology D counter was well appreciated and briefly discussed after the platform presentation.

The third chapter, entitled "**Validation of counting and chromatic characterization in ecotoxicological tests: D counter**" provides a comparison between the traditional approach and the innovative technology considering a bioassay, where a chronic test (21 days) was performed with *Daphnia magna*, without exposure to chemicals, with the main objective of assessing the accuracy and precision of the new equipment. In this study, the D counter was equipped with the LASER lights module.

The fourth chapter, entitled **"Combined effects of ammonia and hardness on *Daphnia magna*"** describes a typical ecotoxicological test but enhanced with D counter performance, providing information about combined effects of water hardness with ammonium chloride (NH_4Cl) on growth, reproduction and population growth of *D. magna*. For this, the efficiency of the new equipment was analysed, comparing with the classic tests of ecotoxicology and the new methodology was applied in a classic ecotoxicology test. The D counter was equipped with the LED lights module.

The last chapter overviews results obtained in the present study and discusses the validation of the new equipment D counter, for counting and discrimination of organisms applied in a case study using *D. magna* as model organism.

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Chapter 2

D counter: Automatic Organism Counting and Characterization in Ecotoxicology assays

D counter: Automatic Organism Counting and Characterization in Ecotoxicology assays

Abstract

The chronic toxicity test with the microcrustacean *Daphnia magna* is one of the most commonly used tests in aquatic Ecotoxicology. This test requires the counting of the number of offspring produced by daphnids for 21 days. Currently, the counting is performed by technicians, manually, in backlight conditions. However, given the number of treatments and replicates per treatment, the counting procedure is not only time consuming, but also very susceptible to human error and even to visual impairment of the technician. Therefore, the development of a device for automatic counting is of large importance. Following this line of thought, we present a device for automatic counting of *Daphnia* offspring, which can also estimate *Daphnia* body length – the D counter. In each test, the current version of the device forces the passage of all the organisms through a group of optical sensors. The acquired digital signals are then processed in real time by a computing system in order to automatically count and characterize each organism. All the acquired data is stored allowing it to be reprocessed using different detection and characterization criteria and to be exported in common data processing applications (such as Excel).

To validate the estimation of the daphnids BL a short test was carried out using daphnids with varying sizes (0-21d old) (three replicates per age). Each daphnid passed through the D counter 3 times. The daphnids BL was measured under a stereomicroscope, and compared to the values obtained by the D counter.

Considering the estimation of daphnids BL, the values obtained by the D counter correlate well with the daphnids BL measured under a stereomicroscope (linear regression n=33, r^2 adjusted=0.900, F= 289.6996, p<0.0001).

Regarding the life table experiment, no significant differences were observed between the counts of both technicians (Mann-Whitney U Statistic= 6726.000, T=13512.000, n=136, p=0.998). For this reason, these counts were averaged and assumed as the manual counting data set. In parallel, the number of offspring

obtained using D counter device was similar to the manual counting data set, as illustrated in figure 6. (Mann-Whitney U Statistic= 6568.000, T= 13674.000, n= 136, p= 0.755). The obtained regression (n=136, r^2-adjusted=0.920, F= 1588.1205, p<0.0001), corroborates that manual and D counter counts are not statistically different.

Overall results show the lack of significant differences between the manual and the D counter estimated values concerning both BL and number of offspring. The D counter device is a useful tool to be applied in ecotoxicology assays involving *Daphnia* sp., or other aquatic organisms such as *Arthemia* sp. Preliminary tests also showed its application in *Danio renio* eggs differentiation and counting.

Keywords: Automated counting, Bioassays, D counter, *Daphnia magna*, Ecotoxicology, innovative technology

2.1 Introduction

Ecotoxicology assays require the handle of specific organisms with ecological relevance. The OECD (Organization for Economic Cooperation and Development) or ISO (International Organization for Standardization) has standardized a series of toxicity tests for ecological risk assessment using several different key species (Connon et al. 2012). Among aquatic organisms, the genus *Daphnia*, namely the species *Daphnia magna*, is commonly used as a standard species in Ecotoxicology, being recommended as a model species by the organizations mentioned above.

The chronic toxicity test with the microcrustacean *Daphnia magna* is one of the most commonly used tests in aquatic Ecotoxicology. This test requires the counting of the number of offspring produced by daphnids for 21 days. Currently, the counting is performed by technicians, manually, in backlight conditions. However, given the number of treatments and replicates per treatment, the counting procedure is not only time consuming, but also very susceptible to human error and even to visual impairment of the technician. Therefore, the development of a device for automatic counting is of sum importance. Following this line of thought, we present a device

for automatic counting of *Daphnia* offspring, which can also estimate *Daphnia* body length – the D counter (figure 4). In each test, the current version of the device forces the passage of all the organisms through a group of optical sensors. The acquired digital signals are then processed in real-time by a computing system in order to automatically count and characterize each organism. All the acquired data is stored allowing it to be reprocessed using different detection and characterization criteria and also to be exported in common data processing applications (such as Excel).

Figure 4- D counter.

2.2 Materials and methods

To validate the estimation of the daphnids BL a short test was carried out using daphnids with varying sizes (0-21d old) (three replicates per age). Each daphnid passed through the D counter 3 times. The daphnids BL was measured under a stereomicroscope, and compared to the values obtained by the D counter.

To validate the automatic counting we carried out a 21-d life table experiment with *D. magna*. The experimental procedure followed the OECD guideline 211 (OCDE 2008) but no toxic chemicals were used. Briefly, neonates (less than 24 h) were individually placed in glass vials containing 50 ml of ASTM hard water containing a standard organic additive and the algae *Pseudokirchneriella subcapitata*. The temperature was 21±1°C and the photoperiod 16h:8h (light:dark), similarly to the culture conditions. Thirty replicates were used. Daphnids were fed every day and the test medium was renewed every other day.

During the media renewal, the offspring were counted twice, manually by two technicians. After “manual” counting, the content of each vial was passed twice through the D counter, for the automatic counting of the offspring. The results of the manual and automatic counting were compared. Statistical analyses were carried out using SigmaPlot v12.5, using a significance level of 0.05.

2.3 Results and discussion

Considering the estimation of daphnids BL, the values obtained by the D counter correlate well with the daphnids BL measured under a stereomicroscope (figure 5) (linear regression n=33, r^2 adjusted=0.900, F= 289.6996, p<0.0001).

Regarding the life table experiment, no significant differences were observed between the counts of both technicians (Mann-Whitney U Statistic= 6726.000, T=13512.000, n=136, p=0.998). For this reason, these counts were averaged and assumed as the manual counting data set. In parallel, the number of offspring obtained using D counter device was similar to the manual counting data set, as illustrated in figure 6 (Mann-Whitney U Statistic= 6568.000, T= 13674.000, n= 136, p= 0.755). The obtained regression (n=136, r2-adjusted=0.920, F= 1588.1205, p<0.0001), corroborates that manual and D counter counts are not statistically different.

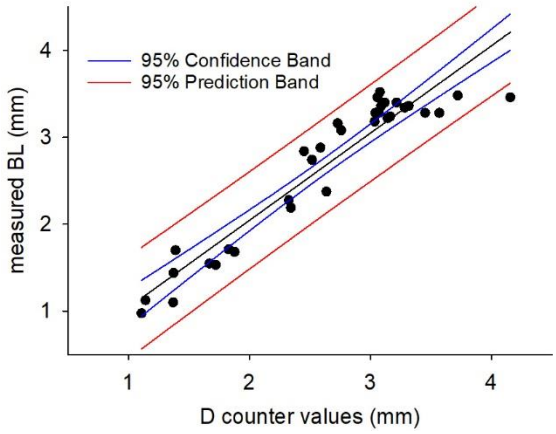

Figure 5- Body length (BL): D counter vs measured body length. Prediction and Confidence bands (95%).

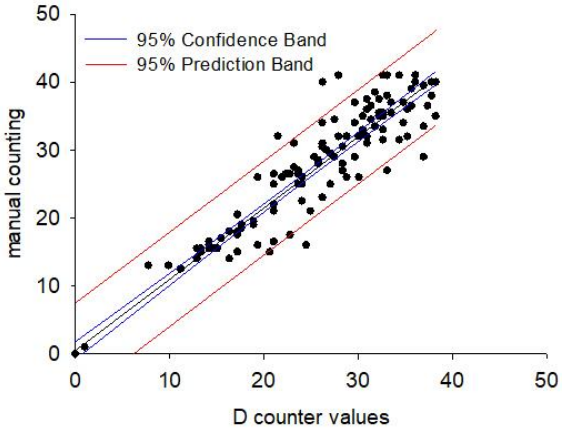

Figure 6- Offspring counting: D counter vs manual counting. Prediction and Confidence bands (95%).

2.4 Conclusions

Overall results show the lack of significant differences between the manual and the D counter estimated values concerning both BL and number of offspring. Nevertheless, further research is being carried out, in order to establish the limits of this device and the possibilities of upgrading it in order to improve its predictive capacity and application in scientific testing.

The D counter device is a useful tool to be applied in ecotoxicology assays involving *Daphnia* sp, or other aquatic organisms such as *Arthemia* sp. Preliminary tests also showed its application in *Danio renio* eggs differentiation and counting.

2.5 Bibliography

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Chapter 3

Validation of counting and chromatic characterization in ecotoxicological tests: D counter

Validation of counting and chromatic characterization in ecotoxicological tests: D counter

Abstract

D counter is an automatic counting and chromatic analyses device, able to count and measure high quantities of microorganisms usually used in ecotoxicological tests. This device becomes a promising innovation due to the fact that it allows for a faster and more efficient way to collect data, avoiding some errors present in the classical method. This study provides a comparison between the classical approach and the innovative technology considering a bioassay, where a chronic test (21 days) was performed with *Daphnia magna*, without exposure to chemicals, with the main objective of assessing the accuracy and precision of the innovative device, D counter.

The results have shown that the new device can effectively assist or even replace the counting and characterization of organisms, currently still performed by laboratory technicians. This equipment has shown throughout the test that it is possible to save work time, increase productivity. The values resulting from the simulation of the chronic test, did not show significant differences between manual and automatic counting ($p=0.453$), considering the estimation of daphnids BL, the values obtained by the D counter correlate well with the daphnids BL measured under stereomicroscope ($p=0.471$).

Key words: Counting, Measuring organisms, New method, Ecotoxicological tests, *Daphnia magna*

3.1 Introduction

The D counter is an automatic counting and chromatic analyses device able to deal with both microorganisms and particles in suspension in aquatic medium, ex. *Daphnia sp*. as well as others with reduced dimensions. The device is an asset for ecotoxicological tests, due to its ability to automatically count high quantities of microorganisms, and measure their body length (BL) (Oliveira e Silva et al., 2015). This device becomes a promising innovation due to the fact that it allows for a faster and more efficient way to collect data, avoiding some errors. Hence, there are a few limitations to these tests that can cast a reflection on the results obtained. Most of these ecotoxicological tests require the counting and characterization of various organisms exposed to different concentrations. However, this process becomes very tiring for the operator, not only because each organism is counted one at the time, but also since it can cause damages in the visual acuity of the operator, not ruling out the possible human error in counts and exposure to chemicals. Therefore, different concentrations of the chemical in a study will require the counting process to be repeated for each replica, where the number of organisms being used at each test concentration is reduced from at least 40, preferably divided into four groups of 10 organisms (OECD 2008).

The most common ecotoxicological tests with *Daphnia sp.* are chronic tests. Commonly, chronic tests with *D. magna* last for 21 days and require observation of the test organisms every other day, as well as the registry of the number of offspring produced and the final size of the test organisms (OECD 2008), thus, allowing the determination of the effects of the chemical substance on reproduction and growth of *Daphnia.* To minimize the possible inherent errors in a manual count and to optimize the count of the microorganisms, the present work aims to validate the D counter. To achieve this goal, we carried out a life-table experiment, similar to a chronic test, but with no toxic chemicals. During this experiment, both counting methods - manual and automatic - were performed and posteriorly compared. In other experiment, the body size of the test organisms was estimated using the

automatic device and compared to the size determined using a stereomicroscope. This is the first time an automatic counting device is used for counting *Daphnia*, without estimates, but effectively counting each organism in suspension.

3.2 Materials and Methods

3.2.1 Counting test

All experiments were performed with a laboratorial culture of the water flea *D. magna* (clone Beak). Cultures were maintained in ASTM hardwater (ASTM 2004) enriched with a standard organic additive (300µl/50ml) (Marinure seaweed extract, Glenside Organics Ltd., UK). They were fed half the food in the first week, and in the following weeks with the recommended total daily allowance, with *Raphidocelis subcapitata* (3.0×10^5 cells/ml). Culture medium was renewed every other day. The culture was kept at 20±2°C under a light (16h): dark (8h) photoperiod.

Two independent tests were carried out, with 15 replicates each. Tests were similar, but one of these tests was used to obtain data to adjust the counting procedure and the other one was used to obtain data for validation. Each test initiated with fifteen neonates (less than 24h). Tests followed the OECD guidelines N211 (OECD, 2008) with the exception that no toxic chemicals were used. The exposure conditions were similar to the culture conditions mentioned above. Neonates were kept individually in glass vials containing 50ml of test medium, for 21 days. Daily, test organisms were checked for mortality, molts and reproduction. Medium was renewed every other day. Previously to medium renewal, the content of each glass vial (including the daphniid) was passed through the D counter, 2 times each. After the start of reproduction, this automatic counting was preceded by manual counting. The manual counting was performed by two technicians, in back-light conditions, using a plastic pipette and counting the juveniles as they were removed from the test medium.

Manual counting was conducted in order to assess whether or not there were significant differences between these two counting methods.

3.2.2 Size test

All experiments were performed with a laboratorial culture of the water flea *D. magna* (clone Beak). Cultures were maintained in ASTM hardwater (ASTM 2004) enriched with a standard organic additive (300µl/50ml) (Marinure seaweed extract, Glenside Organics Ltd., UK). They were fed with with *Raphidocelis subcapitata* (3.0 x 10^5 cells/ml. The culture was kept at 20±2°C under a light (16h): dark (8h) photoperiod.

The cultures were kept through several generations of daphnids, 3 replicas each: (A1, A2, A3), in 50 ml glass vials, according to the conditions followed OECD guideline, mentioned above (OECD 2008).

The present experiment was made just in one day, and aimed for the comparison of BL (Body length) measured in the D counter and manually. Each glass vial was passed 3 time through the D counter, and the size of each organism was measured under a stereomicroscope (MS5, Leica Microsystems, Houston, TX, USA), with the aim of certifying whether or not there were significant differences between the results obtain in the two counting methods.

3.2.3 Data treatment and statistical analyses

All statistical analyses were performed using the SigmaPlot statistics package (SigmaStat 3.5, SPSS Inc.USA), with a significance level of 0.05.

To compare the countings of both technician a paired T-test was used. Since there were no significant differences between the coutings of both technicians, the average was determined and compared to the D counter countings using a paired T-test.

Concerning the size test, the values obtained using the stereomicroscope were compared to the values obtained with the D counter using also a paired T-test.

3.3 Results and Discussion

3.3.1 Counting test

Since the counting of juveniles was performed every 48 h, it sometimes occurred in the presence of molts.

Concerning the manual counting procedure, no significant differences were observed between the two independent countings obtained by the technicians (paired-T test, p=0.839). For this reason, the values of both technicians were averaged and compared to the values of D counter. This comparison also showed no significant differences between manual and automatic counting (paired-T test, p =0.453).

The matching between manual counting (average of both technicians) and automatic counting is depicted in figure 7.

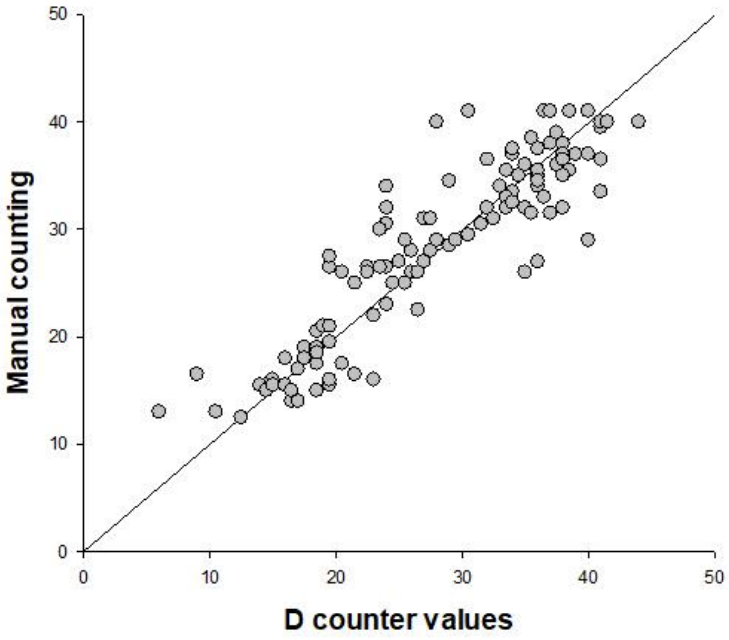

Figure 7- Matching of values between manual counting and D counter.

Regarding the regression of both counting techniques, it is defined by the following equation: Number of juveniles= 0,9977 X D counter. There is a good correlation between the values of manual counting and D counter values, as given by the fitting parameters of the regression: r^2= 0.79, n= 116, p= <0.0001.

The automatic counting of the organism is much faster (30s) than the manual counting (>60s), and it does not cause visual fatigue. Another great advantage of this equipment is that it allows endless re-countings of the same vial, processing several repetitions in a short time. These advantages, allied to the results obtained by comparing the two counting methods, suggest that this equipment could replace manual counting in a very effective and reliable way, thus can be an useful tool in ecotoxicology assays, eg. for all those who work with *D. magna* and other small organisms with similar characteristics.

3.3.2 Size test

Throughout the test, the daphnids size was estimated based on the length of the organisms (see section 3.2.2) and compared to the values given by the D counter. Considering the estimation of daphnids BL, the values obtained by the D counter correlated well with the daphnids BL measured under stereomicroscope (paired-T test, $p = 0.471$).

The matching between manual measured BL and automatic measured BL is depicted in figure 8.

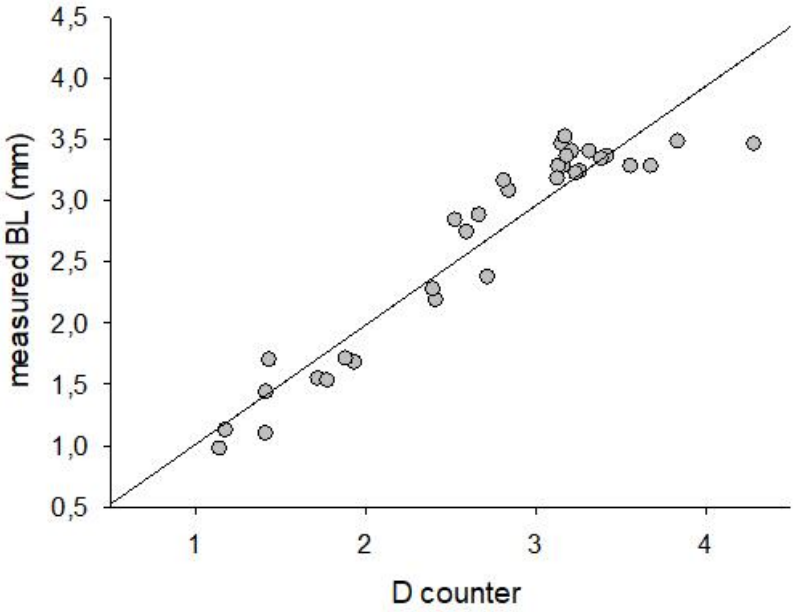

Figure 8- Body length (BL): D counter vs. measured BL.

Regarding the regression of both measurement techniques (Figure 8, it is defined by the following equation: Length-stereomicroscope = 0.0362 + 0.974 x D counter. There is a good agreement between the values of manual measurement and D counter, as given by the fitting parameters of the regression: r^2= 0.90, n= 33, p= <0.0001.

The measurement made by stereomicroscope tends to be time consuming and tiresome. These results suggest that D counter can replace the body lengths measurement with the stereomicroscope in a very positive away, saving time and work.

3.4 Conclusion

In this chapter of the thesis we analyzed the effectiveness of the new equipment (D counter) for the counting and chromatic characterization of organisms such as *D magna*. Overall data indicate that D counter can effectively assist or even replace the classical methods of counting and characterization of organisms such as body length measurements, currently needed in ecotoxicology and other bioassays. D counter has shown to be a useful tool, minimizing effort and being able to eliminate

the most common sources of human error and subjectiveness present in the classical methods.

This device has in fact shown, throughout the test, that it is possible to save work time, increase productivity, give us trustworthy values, and avoid prolonged exposure to chemicals. However, there is still room for the improvement of the device and so, it would be of large importance to proceed with further work. For that reason, in the following chapter, it will be performed a classical Ecotoxicology assay using D. magna exposed to combined contaminant effects, but being the manual counting and human eye observation replaced by D counter counting and chromatic characterization.

3.5 Bibliography

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OECD (2008). OECD Guidelines for the Testing of Chemicals. Test No. 211: *Daphnia magna* Reproduction Test. Paris, Organization for Economic Co-operation and Development: 23.

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Chapter 4

Combined effects of ammonia and hardness on *D. magna*

Combined effects of ammonia and hardness on *Daphnia magna*

Abstract

Ammonia is one of the most used components in agriculture for the manufacturing of fertilizers. However, little is known about the effects it has on *Daphnia magna*. Moreover, little is known regarding its effects on both soft and hard waters. Water hardness is a factor with great impact on aquatic species, and the sub-phyla Crustacea is one of the most affected by this Ca fluctuations. Thus, the main objective of this study was to assess the combined effects of ammonium chloride NH_4Cl (2, 12 and 20 mg/L of NH_4Cl) and water hardness (low and high hardness) on the model organism *D. magna*. Life table experiments were carried out and the endpoints growth, reproduction and feeding rate (FR) were assessed using the device D counter. The results showed that hardness caused major effects on growth and development of *D. magna*: hard water lead to increased growth of daphnids. The effects of NH_4Cl concentration were dependent on the water hardness, being more evident in soft water. In soft water, increased NH_4Cl concentration lead to increased BL. In opposition, in hardwater, there is no visible effect on the variation of BL. Regarding reproduction, the hardwater medium caused a higher rate of reproduction than that observed in the soft water medium. Concerning NH_4Cl concentration, unexpectedly, higher concentrations induced higher rates of reproduction, in both test media. The results highlight the importance of studying the influence of environmental factors on the toxicity of chemicals to aquatic organisms.

Keywords: Ammonia, Hardness, Reproduction, Body length, D counter, *Daphnia magna*

4.1 Introduction

The constant anthropogenic activities provoke the pollution of the environment, which consequently affects the stability of the ecosystems causing, possibly, long term damage. The release of these compounds into surface water occurs mainly due to industrial or agricultural activities, such as fertilized farmlands, areas of concentrated livestock production, or effluents of waste water-treatment (U.S Department of health and human Services 2004). Ammonia (NH_3) is a colourless gas naturally produced. Ammonia can be found naturally in water, soil, air, and its presence is not harmful to humans, because ammonia is an essential compound and source of nitrogen for plants and animals. The biggest problem lies in the ammonia produced by humans, most often resulting from anthropogenic activities, which equals that produced by nature. The large percentage (90%) and most significant use of ammonia and ammonium compounds is for the production of fertilizers (Kramer 2004). Aquatic systems represent the major concern regarding ammonia toxicity. This fact is due to the constant release of both biological waste and runoffs, from urban and agricultural origins, into the aquatic systems. Thus, regions of high human habitation and/or elevated numbers of cattle industry, are the most problematic regions (Randall & Tsui 2002). Hence, aquatic toxicity caused by ammonia is a big issue, however, there is still a considerable lack of knowledge on the way this compound affects the water flea microcrustacean *Daphnia magna* (Ren et al. 2015), a model species in Ecotoxicology.

Ammonia may react with other substances (H_2SO_4, HCl, HNO_3, or N oxides) and form new ammonium compounds, including salts such as ammonium chloride, ammonium sulphate, ammonium nitrate, and others (U.S Department of Health and Human Services 2004). In the specific case of ammonium chloride (NH_4Cl), which is the chemical used in this study, it results from the reaction of ammonia with hydrochloric acid (HCl) and has been used over the years as a compound for the production of chemicals, pharmaceuticals or used like fertilizer in agriculture (Ishikawa et al. 2015; Chang & Chung 2000).

Aquatic food webs have been under a lot of stress due to the cascade impact of recent changes in the water chemistry, whether we're considering marine or

freshwater systems (Betini et al. 2016). The chemical properties of freshwater vary widely, both geographically and temporally, and are often affected by anthropogenic activities, which can also speed up natural processes, like acidification (Cairns & Yan 2009). Acidification is primarily caused by the conversion of sulphur dioxide (SO_2) and nitrogen oxides (NOx), into sulfuric and nitric acids. These two chemicals, can be taken for long distances (Galloway 1995), by the wind, ending up falling of the atmosphere, whether in the dry (acidic gases and particles) or wet (rain, snow, fog and dew) form. Long term of acid deposition can result in the depletion of calcium.

Water hardness is another factor with great impact on aquatic species, and is related to the presence of dissolved calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn) and strontium (Sr). The sub-phyla Crustacea, is one of the most affected by this Ca fluctuations. Their cyclic molts, make them highly dependent on the constant uptake of Ca (Giardini et al. 2015). Aquatic crustaceans have a calcified exoskeleton that is replaced regularly as they grow and a high proportion of the body Ca is lost with the shed molts (Alstad & Hessen 1999). Hence, a decline of calcium ions, will negatively influence the growth, development and survival of many freshwater organisms (Betini et al. 2016). Therefore, the genus *Daphnia* is directly influenced by the water chemistry and, particularly, by the Ca fluctuations. This factor makes *Daphnia* an ideal organism for the evaluation of water chemical parameters. Many studies have already been conducted in order to assess the effects of Ca variations (e.g. Alstad & Hessen 1999) in *Daphnia* life history. However, little is known about the interaction of chemicals, like ammonia, with water hardness and its effects on *Daphnia* species.

Thus, the main objective of this work was to clarify the effect of variation of water hardness, in the presence of different ammonium chloride (NH_4Cl) concentrations (2, 12, 20 mg/l), on *Daphnia magna*. The model species *D. magna* was used as a representative of aquatic crustaceans.

This study was performed using the D counter, a device that performs automatic counting of *Daphnia* offspring and it can also estimate the individual offspring body length (BL) (Oliveira e Silva et al., 2015). In a previous work the application of this device D counter was validated (under submission).

In this paper, we present a work assisted by the new D counter performing a classical bioassay implying counting and measuring daphnids, as well as measuring the medium absorbance to quantify daphnids assimilation along the bioassay. The main parameters studied were the variation of ammonia and hardness and their effect in reproduction, growth and feeding rate (FR) in *D. magna*.

4.2 Materials and Methods

4.2.1 Test organisms

All experiments were performed with a laboratorial culture of *D. magna* (clone Beak). The culture was kept at 20±2°C under a light (16h): dark (8h) photoperiod, and all organisms were fed with *Raphidocelis subcapitata* (3.0 x 10^5 cells/ml), and with a standard organic additive (Marinure seaweed extract). Culture medium was renewed every other day.

A culture of juvenile daphnids was divided in two groups. One group was kept under standard conditions, being cultured in ASTM hardwater (ASTM 2004). The other group was kept under similar conditions, with the exception that organisms were cultured in a soft water medium. This medium was prepared with the same stock solutions as ASTM hardwater, but their concentration was only ¼ of its concentration in the ASTM hardwater. For a characterization of this medium refer to Table 1.

Following the standard procedures, the first three batches of neonates (corresponding to the first three broods) were discarded. The organisms used for the experiment were those from the fourth brood. This procedure was selected to ensure that the neonates used in the experiments were exposed to the test conditions during their entire developmental period, according to Barata and Baird (2000).

4.2.2. Experimental design

The test began with neonates (less than 24h), from parental daphnids properly adapted to ASTM or the soft water medium, following the OECD guideline N211 (OECD 2008). Neonates were transferred to the respective medium with different concentrations of NH_4Cl: 0, 2, 12 and 20 mg/l, using 10 replicates per treatment.

During 21 days, the neonates were individually maintained in glass vials, containing 50 ml of test medium. Conditions such as temperature, photoperiod and seaweed extract, were maintained as described for culturing. Test media were renewed every other day. The organisms were fed half the food in the first week (1.5×10^5 cells/ml of *R. subcapitata*), and in the following weeks with the recommended total daily allowance. Daily, test organisms were checked for mortality, molts and reproduction. After molting, random shedded carapaces were collect for further measurement, in order to determine the daphnids body length (BL). This parameter (from head to the base of spine) was estimated based on the length of the first exopodite of the second antennae (AL), which was measured in the carapace released at the end of each instar. The following equation was used: $BL = 10.98 \times AL - 0.55$ ($r^2=0.978$, n=128, $p<0.0001$). AL was measured under a stereomicroscope (MS5, Leica Microsystems, Houston, TX, USA) with a built-in calibrated eyepiece micrometer. After the first brood, all the vials were counted, on the D counter, three times each. At the same time, half of the test vials were counted manually for comparison of both methods.

4.2.3 Feeding inhibition tests

In order to determine whether or not the feeding activity of daphnids was affected by the experimental conditions, the FR of the organisms, in both test media exposed to the tested NH_4Cl concentrations, were measured. The FR was measured by two different methods: in spectrophotometer (absorvance at 440nm) and in the D counter (at 450nm).

Individuals from parental daphnids adapted to each test medium were selected for the test. Tests were conducted with juveniles from the fourth instar (96h old), using five replicates per treatment. Each replicate consisted of 3 daphnids in 50 ml of the corresponding medium with different ammonia concentrations, as used in the

chronic experiment: 0, 2, 12 and 20 mg/l of NH_4Cl and an algae concentration of $3.0x10^5$ cells/ml per treatment. In addition, 4 blanks (containing algae but no daphnids) were carried out.

In order to avoid algae growth, all the test vials were kept in the dark, at 20 ± 1 ºC for 6h (Jesus et al. 2013). Before the measurement, by spectrophotometry and by the D counter, each vial was vigorously shaken and all the organisms were taken from the vials.

Using the following equation (Allen et al 1995), the FR were determined, using the previously conversion of the absorbance (spectrophotometer) into to cell concentration values:

$$F = \frac{V \times (C_j - C_i)}{n \times (t_j - t_i)}$$

Where F represents the FR of the organisms (cells/animal h); V stands for the volume of medium in each test vial (ml); Ci is the cell concentration at time i and Cj is the cell concentration at time j; n is the number of daphnids; tj is the final time of the exposure and ti is the initial time of exposure.

4.2.4 Chemical analyses

Principal anions and cations (Ca^{2+}, Mg^{2+}, Na^+, CO_3^{2-}, SO_4, K^+, Cl^-) were measured by ion chromatography, using a Dionex IC-5000$^+$ DC equipment. Cations were measured with the column Dionex IonPac CS16- RFIC Analytical 3x250mm, and anions were measured with the column Dionex IonPac AS11-HC-4um Analytical 2x250mm. The solvent used for anions was Potassium hydroxide (KOH, 30Mm), except for the carbonate, which solvent was Potassium hydroxide (KOH, 20mM); for the cations, the solvent was methylsulfonic acid (CH_3SO_3H, 30mM).

Ammonia were analyzed in Hach DR2000 spectrophotometer (Germany), using Nessler reagent, dispersing agent Alcohol Polyvinyl and mineral stabilizer (See annex 1 for a photo of the experimental procedure).

4.2.5 Data treatment and statistical analyses

Sigma Stat 3.5 statistical package was used for statistical analyses. To assess the combined effects of ammonia and hardness in reproduction, growth and FR two-way ANOVA analysis was used. Within each medium, the effect of ammonia concentration was assessed by a one-way ANOVA, and multiple comparisons were performed by the Tukey Test. Data which did not verify normality (Shapiro-Wilk test) and/or homogeneity of variances were analyzed with the nonparametric Kruskal-Wallis test, followed by the Dunn's test for multiple comparisons.

4.3 Results

4.3.1 Reproduction

The combined effects of water hardness and NH_4Cl in the AFR (age first reproduction) and SFR (size first reproduction) of daphnids are depicted in figure 9.

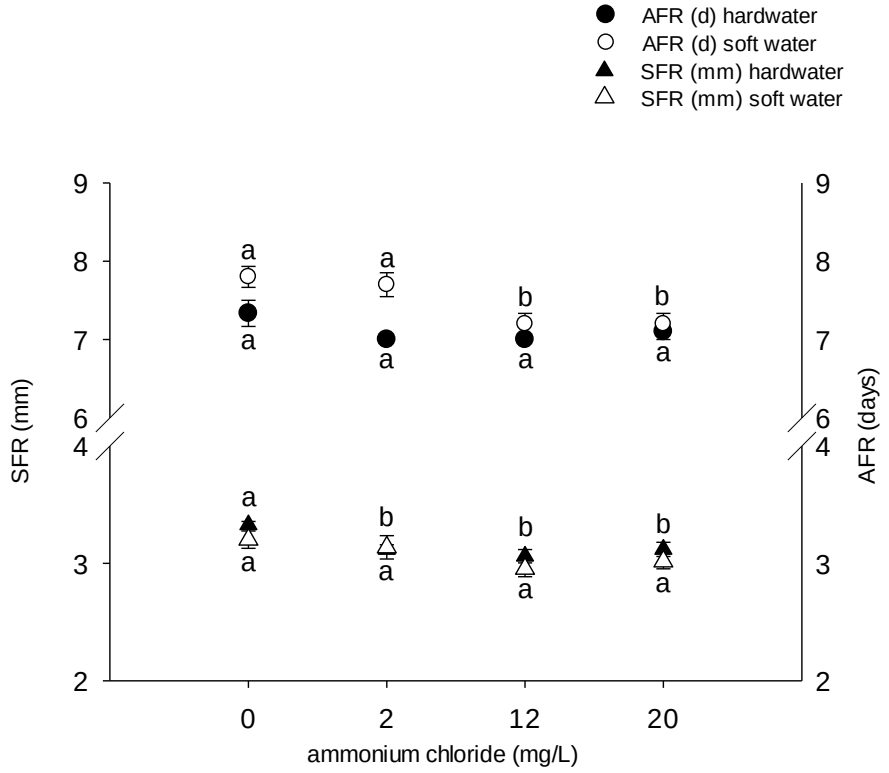

Figure 9- SFR (size first reproduction) and AFR (age first reproduction) of *Daphnia magna* exposed to different concentrations of NH_4Cl in hardwater and soft water. For each medium, different letters stand for significant differences among NH_4Cl.

Hardness and NH_4Cl concentration significantly affected daphnids AFR (F_{medium}= 19.214, df=1, p<0.001; $F_{ammonium}$= 6.366, df=3, p=0.001).

In general, daphnids maintained in soft water were older at first reproduction than the ones in hardwater. In soft water, there was a trend for decreasing AFR with increasing NH_4Cl concentration (p=0.007), whereas in hard water, the NH_4Cl concentration did not significantly affect AFR (p=0.063). However, the interaction between hardness and NH_4Cl was not significant (p=0.055).

Hardness did not significantly affect SFR (F_{medium}= 1.728, df= 1, p=0.193); However, NH_4Cl concentration significantly affected daphnids SFR in hardwater, leading to a trend for decreasing SFR with increasing ammonia concentration; $F_{ammonium}$= 4 df= 40.703, p=0.004).

The combined effects of water hardness and NH_4Cl in the total reproduction of daphnids are depicted in figure 10. The comparison between the manual and automatic counting procedures are shown in Supplementary material (Figure S4.1).

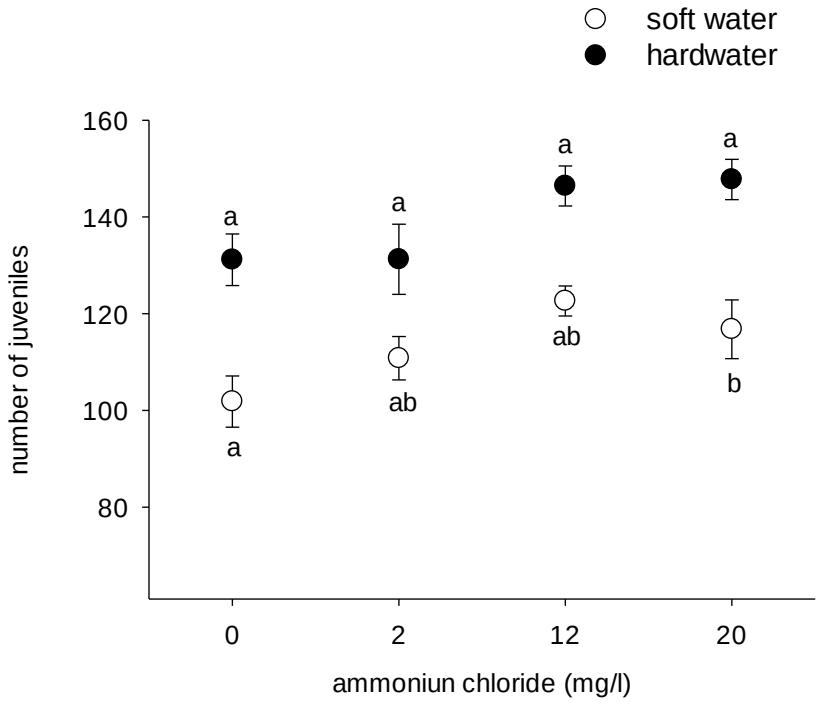

Figure 10- Total reproduction of *Daphnia magna* exposed to different concentration NH_4Cl in ASTM hardwater (filled circles) and ASTM soft water (open circles). For each medium, different letters stand for significant differences among NH_4Cl.

Daphnids reproduction was higher in hardwater than soft water, which highligths a significant effect of water hardness on reproduction (F_{medium}= 52.716, df=1, p<0.001). Concerning the effect of NH_4Cl, increased values resulted in higher reproduction rates in both media ($F_{ammonium}$= 5.818, df=3, p=0.001). However, the interaction between hardness and NH_4Cl was not significant (p=0.713).

4.3.2 Growth

The combined effects of water hardness and NH_4Cl in the BL of daphnids are depicted in figure 11. The comparison between the manual and automatic counting procedures are shown in Supplementary material (Figure S4.2).

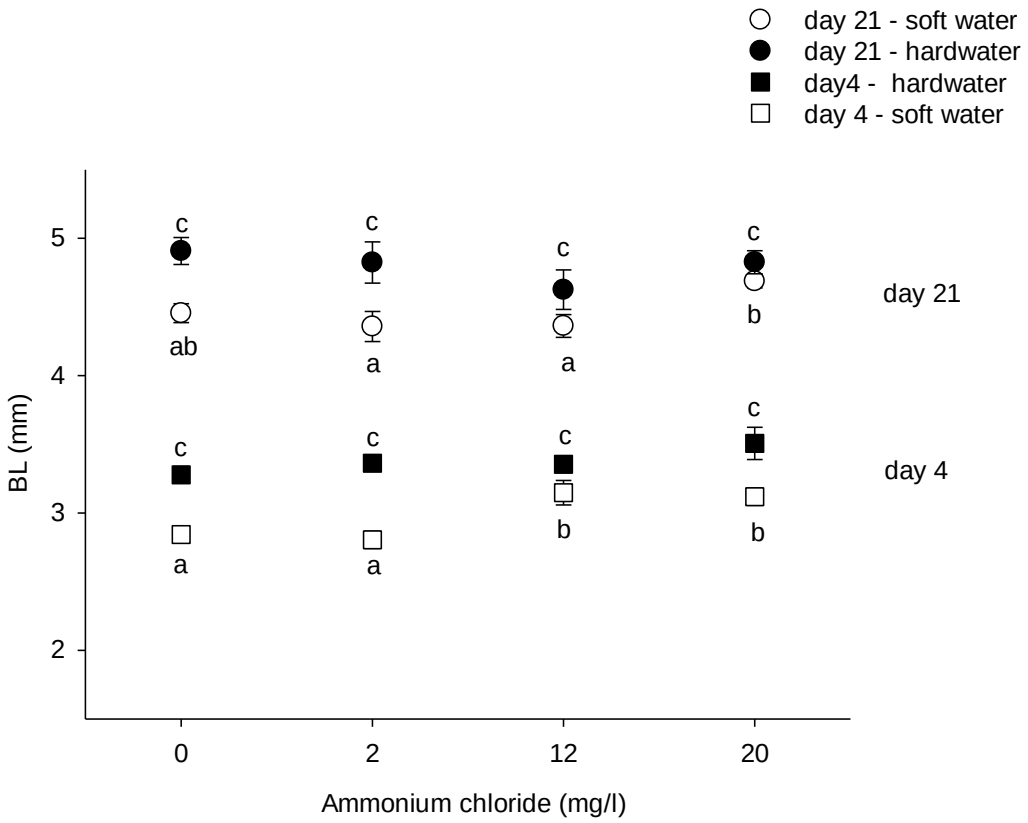

Figure 11- BL of *D. magna* exposed to different concentration of NH_4Cl in ASTM hardwater (filled circles) and ASTM soft water (open circles), both at day 4 and day 21. For each medium, different letters stand for significant differences among NH_4Cl.

Either at day 4 or day 21, daphnids body length (BL) in hardwater was higher than in soft water, showing a significant effect of water chemistry, $p<0.001$.

At day 4, both water chemistry and NH_4Cl concentration significantly affected daphnids BL ($F_{medium}= 59.499$, df=1, $p<0.001$; $F_{ammonium}= 5.878$, df=3, p=0.001).

Concerning NH_4Cl concentration, there was a significant difference ($p<0.001$) between the lowest (0 and 2 mg/l) and the highest concentrations (12 and 20 mg/l) in soft water. On the other hand, in hardwater, NH_4Cl concentration had no significant effect (p=0.359) on daphnids BL at day 4.

Daphnids in ASTM hardwater were higher than those living in the soft water medium: in hard water BL varied between 2.92 and 4.49 mm, whereas in soft water BL varied between 2.47 and 3.39 mm.

At day 21 water chemistry significantly affect daphnids BL ($F_{medium}=20.544$, df=1. $p<0.001$). The same is not observed in effect of NH_4Cl concentration, had no significant effect on daphnids BL ($F_{ammonium}=0.845$, df=3, p=0.481) at hardwater medium. However, in the soft water medium, the effect of NH_4Cl concentration has a significant effect in BL ($F_{ammonium}=3.565$, df=3, p=0.024).

At hardwater medium, the presence of NH_4Cl at higher concentrations, resulted in lower BL values, when compared to those observed at lower concentrations. At soft water, the opposite effect is observable: the presence of NH_4Cl at higher concentrations, resulted in higher BL values, when compared to those registered at lower concentrations.

The BL of daphnids in ASTM hardwater varied between 4.0 and 5.3mm, whereas in the soft water, BL varied between 3.7 and 4.9mm

4.3.3 Feeding inhibition tests

The combined effects of water hardness and NH_4Cl in the FR (feeding rate) of daphnids are depicted in figure 12a and 12b. The comparison between the algae absorbance given by the spectrophotometer at 440 nm and by the D counter is depicted in Supplementary material (Figure S4.3). Given the low correlation between the values given by both techniques, we show the FR of daphnids determined using both techniques.

The combined effects of water hardness and NH_4Cl in the FR of daphnids are depicted in figure 12a and 12b, respectively.

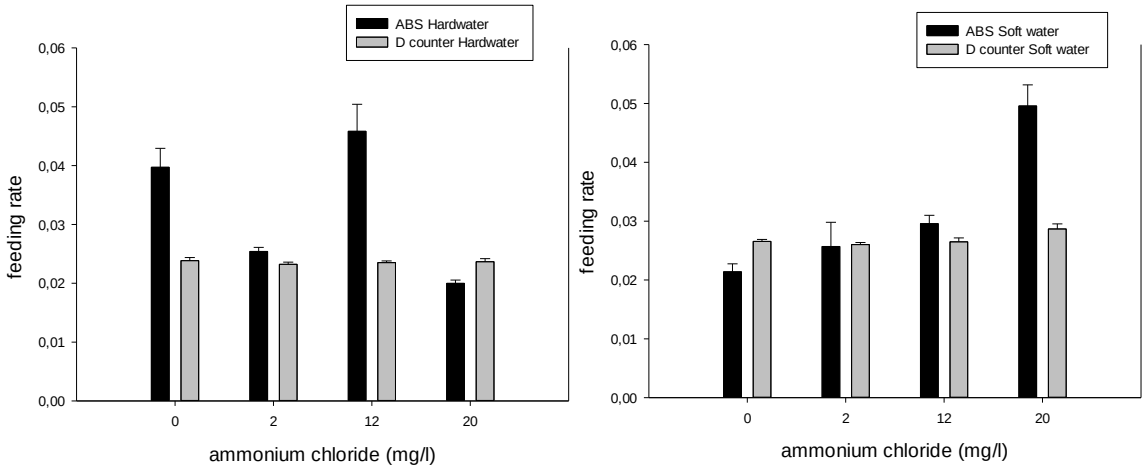

Figure 12A and 12B- **A-** FR (µg dw ind^{-1} h^{-1}) of daphnids exposed to different NH_4Cl concentrations in hardwater, measured both using the spectrophotometer data (black bars) and the D counter data (grey bars). **B-** FR (µg dw ind^{-1} h^{-1}) of daphnids exposed to different NH_4Cl concentration in soft water, measured both using the spectrophotometer data (black bars) and the D counter data (grey bars).

Focusing on the FR values in hardwater obtained by using the spectrophotometer data, there were significant differences due to NH_4Cl concentration ($p<0.001$). Compared to the control, the concentrations 2 and 20 mg/l produced lower FR.

In opposition, the FR values obtained by using the D counter data show no significant differences ($p=0.793$).

Regarding the values of FR in soft water taken by the spectrophotometer, the results show significant differences due to the NH_4Cl concentration ($p<0.001$). When comparing with the control, there are significant differences for the concentrations 0

and 20 mg/l ($p<0.001$). The FR is proportional to the concentration: the higher the concentration, the bigger the FR.

The values measured by the D counter show significant differences ($p=0.025$), between the concentration 2 and 20mg/l.

4.3.4 Quantification of chemicals

The concentration of major ions in both the soft water medium and in the ASTM hardwater is depicted in table 1.

Table 1- Quantification of major ions in both the soft water medium and in the ASTM hardwater (see material and methods for description).

Concentration of major ions (mg/l)	**Hardwater**	**Soft water**
Ca^{2+}	34.25±1.888	13.85±1.294
Mg^{2+}	32.66±5.155	3.49± 0.297
Na^{+}	84.06±7.509	19.42±1.195
$SO_4(2^-)$	154.73±0.997	42.47±2.036
CO_3^{2-}	159.47±0	31.68±0
K^{+}	0.615±0.190	0.545±0.276
Cl^{-}	8.805±0.021	2.5±0.057

The concentration of NH_4Cl in both the soft water medium treatments and in the ASTM hardwater treatments is depicted in table 2.

Table 2- Quantification of ammonium (NH_4^+) in both the soft water and in the ASTM hardwater treatments (see material and methods for description).

Medium	**Nominal concentration of NH_4Cl (mg/l)**	**Expected NH_4^+ concentration (mg/l)**	**Measured NH_4^+ concentration (mg/l)**	
			0h	**48h**
Hardwater	0	0	0±0	0±0
	2	0.67	0.63±0.017	0.48±0.025
	12	4.04	3.61±0.029	3.00±0
	20	6.74	5.20±0.436	5.03±0.058
soft water	0	0	0±0	0±0
	2	0.67	0.56±0.023	0.46±0.012
	12	4.04	3.37±0.076	2.65±0.132
	20	6.74	5.00±0.265	4.43±0.321

4.4 Discussion

In this study, we assessed the combined effects of NH_4Cl and water hardness on *Daphnia magna*. As predicted, and already described by several authors (Hessen et al. 2000; Jesus 2012), the variation of water chemistry effectively affects the development of daphnids. However, there is still a lack of knowledge regarding the effects of ammonia in *Daphnia* and its possible interactions with water hardness.

Reproduction

AFR was reduced with increasing hardness, concordantly with previous studies (Jesus 2012). The effect of NH_4Cl concentration was only significant in the soft water medium, with increasing concentrations leading to reduced AFR. In other words, in the soft water medium daphnids reared at higher NH_4Cl concentrations started reproducing earlier than daphnids reared at lower NH_4Cl concentrations.

Concerning the size at first reproduction, it was affected by NH_4Cl concentration but not by hardness. The fact that SFR was not affected by hardness was unexpected, and disagrees with previous studies (Alstad & Hessen 1999). This might be related to the experimental conditions and/or to the inherent variability of the D counter values for estimating daphnids BL. However, when focusing on the soft water medium, it was observed that the daphnids began to reproduce earlier, at higher NH_4Cl concentrations. This result can be explained considering the fact low concentration of nutrients in the medium (low hardness allied to low NH_4Cl concentration), could act as a deleterious factor, leading organisms to reproduce later (higher AFR). Focusing on the hardwater medium results, and considering the variation of NH_4Cl, the AFR parameter exhibited no significant differences, contrasting with results previously obtained (Ren *et al*. 2015). In this medium, the high concentration of ions might have masked the effect of NH_4Cl. The differences relative to the work of Ren and collaborators (2015) might be related to clonal variability within the *D. magna* species.

Daphnids reproduction was higher in hardwater than soft water, which highligths the significant role of water hardness on reproduction (Jesus 2012). However, the effect of NH_4Cl is also clear: higher concentrations values resulted in higher reproduction rates, in both media. This outcome contrasts with the results described by Ren *et al*. (2015). Indeed, these authors found higher reproduction for *D. magna* exposed to NH_4CL concentrations, in the control and 2mg/l, respectively. The results obtained in the present study can raise questions on whether ammonia acts as an incentive to increase the reproduction rates of these.
These data agree with those of AFR: in soft water, the daphnids maintained at higher concentrations of NH_4CL, presented lower AFR, that is, they began to reproduce earlier and, for that reason, they had higher reproduction rates.

Growth and development

Generally, the results show that the organisms grown in the hardwater medium are bigger that those grown in the soft water medium. This outcome is supported by several authors, stating that decreased hardness conditions are responsible to smaller animal size (Alstad et al. 1999; Hessen et al. 2000).
Water chemistry exhibited a significant influence on the growth of the organisms. In general, the BL of the organisms grown in ASTM hardwater was higher than that of those grown in soft water. This can be explained by the high Ca demands, already described by several authors (Alstad & Hessen 1999). In the hardwater medium, the effect of NH_4Cl concentration on the BL of daphnids at day 21 contrasts with the reported by Ren et al. 2015. Hence, young daphnids (day 4) exhibit no significant differences in BL, and older daphnids (21 days old) show reduced BL at higher concentrations of ammonium chloride (12 mg/l), however this result has no significant value.
Regarding the effects of NH_4Cl concentration on the BL of daphnids reared in the soft water medium, at day 4, there were significant differences between the lowest (2 mg/l) and the highest concentrations of NH_4Cl (12 and 20 mg/L). This result agrees with the results obtained for AFR. Indeed, in the soft water medium, daphnids began to reproduce earlier at higher NH_4Cl concentrations. This highlights

the role of NH_4Cl concentration on *Daphnia* life history in media with low concentration of nutrients. Under these circumstances, NH_4Cl can act as beneficial instead of deleterious. This result is important when considering the effects of NH_4Cl in natural aquatic systems, as it shows that in soft waters concentrations of NH_4Cl in the range tested in this study might improve daphnids growth and reproduction.

Feeding inhibition tests

The correlation between the values obtained by the two methods show an overall reasonable match. Therefore, the FR determined by both methods were presented. The feeding rate values obtained by the D counter do not exhibit significant differences in hardwater. However, the opposite occurred in soft water, where the highest NH_4Cl concentration exhibited a higher FR. Despite this, the values are very similar among all the concentrations and in both media. Hence, these results suggest that the hardness variation and the concentration of NH_4Cl only slightly affected the FR.

Regarding the values obtained by the classic method (spectrophotometer), results show that, in general, the FR of the organisms maintained in soft water are lower than those observed in hardwater. This outcome supports the tendency for the organisms kept in soft water to reproduce later and with lower BL than those of hardwater. Concerning the effect of ammonium, in the soft water medium, the FR are proportional with the increasing concentration of NH_4Cl. These results agree with the BL measurements taken at day 4, and with AFR. However, regarding SFR the result is not proportional to the FR.

On the other hand, in hardwater, there is no observable proportionality in the FR and the concentration of NH_4Cl. In fact, the control and the 12mg/l concentration exhibit higher FR.

Energy uptake is a function of the FR of daphnids. Given that these organisms need energy for growth and reproduction, it was expected a correlation between FR and both growth and reproduction. Thus, the results given by the spectrophotometer would make more sense.

However, further studies need to be carried out to clarify which technique gave more accurate results. Note that even the spectrophotometer values are subject to error, due, for instance, to the conditions of the cuvette used for measurement of the algae absorbance.

It is important to highlight the concordance between the results obtained by the traditional techniques and by the D counter, except for the FR. In fact, the use of D counter allowed a faster and more efficient way to collect data,

Ammonia and ammonium compounds are commonly used in agriculture (U.S Department of health and human Services 2004), which production is not expected to decrease. Thus, it is important to study its effects to aquatic biota. This study suggested that in soft waters, NH_4Cl worked as stimulus for increased BL and reproduction of *D*. *magna*. This would lead to beneficial changes in the aquatic food chains, given the central role of *D. magna* on aquatic food webs (zooplankton grazer). Moreover, these results might be valid not only for *Daphnia*, but also for other crustacean species.

4.5 Conclusion

The present study demonstrated the variety of effects that can emerge from the combination of different stressors that can be found daily in the aquatic environment. In fact, the results obtained corroborated the major effect water hardness has on both growth and reproduction of *Daphnia magna*. In addition, it highlights the relevant role of NH_4Cl concentration particularly in soft water media. In natural soft waters increasing concentrations of NH_4Cl may not act as a deleterious factor, but, instead, improve the growth and reproduction of *D. magna*.

However, the interpretation of combined effects of several variables is still problematic given the lack of data for the comparison of results. This study intended to give rise to new strategies, like the use of the D counter, able to increase both productivity and trustworthiness of the results obtained in ecotoxicological assays.

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Chapter 5

General Discussion

5.1 General Discussion

In this thesis, we approach a new methodology of counting and characterization of microorganisms, D counter. After the presentation of the equipment, its validation was sought, in order to support its reliability in ecotoxicological tests.

The device is an asset for ecotoxicological tests, due to its ability to automatically count high quantities of microorganisms, measure their body length (BL) and algae concentration present in the test medium.

For the validation process, a 21-day chronic test was performed, in the absence of a chemical, aiming to compare the results obtained by the classical counting method to those obtained by the D counter. For this, the model organism chosen was *D. magna*. This microcrustacean is one of the most commonly used test organism in ecological and ecotoxicological studies, and plays a central role in the food web (Lampert 2006).

After validation, the applicability of the equipment was tested in an ecotoxicological test. The main objective of this test was to assess the effect of variation of water hardness (low hardness and high hardness), in the presence of different ammonium chloride (NH_4Cl) concentrations, on the model organism *D. magna.* The importance of these type of studies relies on the variation of several parameters at the same time. Hence, this approach is able to simulate conditions that are often found in natural environments. However, there is still missing information on the effects this variation of combined effects can impact natural ecosystems.

One of the most important, and well documented, parameter for *D. magna* is water hardness. These organisms highly depend on Ca concentration both to grow and reproduce (Ashforth & Yan 2008; Hessen et al. 2000). When in natural environment, these organisms are often under the pressure of several parameters, as mentioned before. The present study chose to focus on the effects that NH_4Cl has, when combined with the water hardness factor. Both ammonia and ammonium compounds are frequently used in agricultural industries, as fertilizers (Kramer

2004). However, and despite their frequent presence in aquatic ecosystems, little is known on its effects on *D. magna*.

Regarding the reliability and potential of the D counter, the results obtained were in line with the expected. This new method proved to be suitable to replace processes currently performed by technicians, that require a lot of time and, due to continuous effort, can induce errors. D counter was created to reduce these errors and automate the process to produce reliable results without the need for much effort from the behalf of the technician. However, the D counter has shown a few issues on its ability to measure the algae concentration within the mediums and, for this reason, further the optimization of this feature is still required.

Regarding the third test results, a few findings were made on the correlation between water hardness and different concentrations of NH_4Cl. The effect of hardness on reproduction was clear: reproduction rates were higher in hardwater than in soft water, being this outcome in agreement with several authors (Jesus 2012; Hessen et al. 2000), and can be explain, in part, by the demand of Ca required by these organisms (Ashforth & Yan 2008; Hessen et al. 2000). However, the effect of NH_4Cl concentrations resulted in higher reproduction rates, in both media. This outcome contrasts with the results described by Ren *et al*. (2015). These data agree with those of AFR: in soft water, the daphnids maintained at higher concentrations of NH_4CL, presented lower AFR, that is, they began to reproduce earlier and, for that reason, they had higher reproduction rates. However, the results obtained in the present study can raise questions on whether ammonia acts as an incentive to increase the reproduction rates of these. Nowadays, most aquatic organisms have to survive and prosper in waters that do not compile the best conditions for their development. Hence, the results illustrate the capacity of organisms to thrive in adverse conditions and, sometimes, their ability to take advantage of certain compounds that are often considered as toxic.

In a general way, the FR of the organisms maintained in soft water are lower than those observed in hardwater. This outcome supports the tendency for the organisms kept in soft water to reproduce later and with lower BL than those of hardwater.

In general, this study has shown the combined effects of water hardness and NH_4Cl on *D. magna,* however these results were not always in agreement with the bibliography. Therefore, further research is required to compliment the data collected. However, the use of data collected from the D counter can introduce some speculation on its reliability because of the fact that it's the first time this equipment is used in a context of bioassays studies. However, several advantages have been demonstrated through this test, in addition to the results obtained by comparing the two counting methods, this equipment has proved to be an ally for all those who work with small organisms.

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Supplementary material

1. Matching between manual counting and automatic counting

The matching between manual counting and automatic counting from results of chapter 3, is depicted in figure S4.1.

Regarding the regression of both counting techniques (figure S4.1), it is defined by the following equation: Number of juveniles=1.5706 + 0.9358 x D counter. There is a good agreement between the values of manual counting and D counter, as given by the fitting parameters of the regression: r^2= 0,9330, n= 192, p<0.0001.

This comparison showed no significant differences between manual and automatic counting (paired-T test, p=0.822).

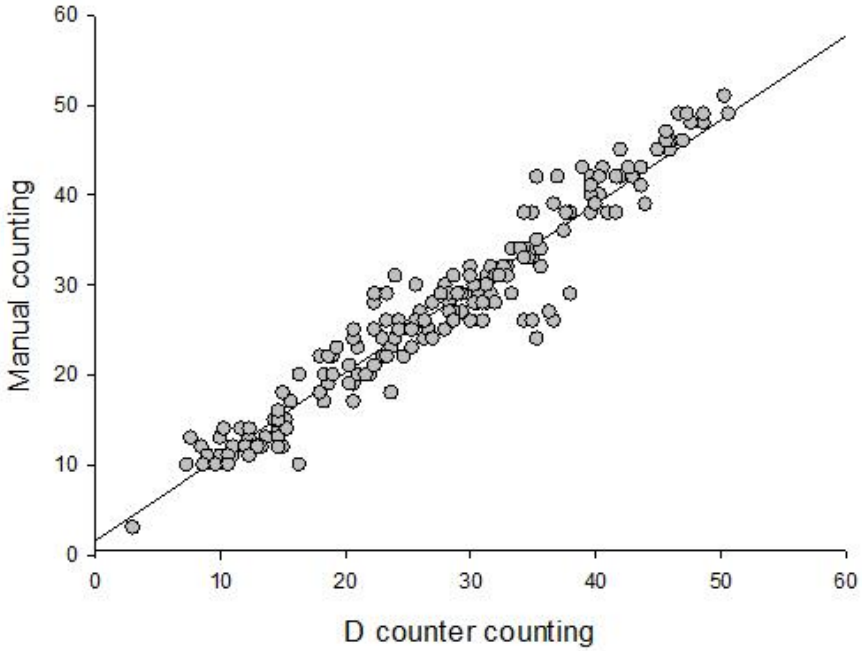


Figure S4 1- Matching of values between manual counting and D counter.

2. Matching between measured BL and automatic measured BL

Considering the estimation of daphnids BL, the values obtained by the D counter correlated well with the daphnids BL measured under stereomicroscope (paired-T test, p = 0.99).

The matching between manual measured BL and automatic measured BL is depicted in figure S4.2.

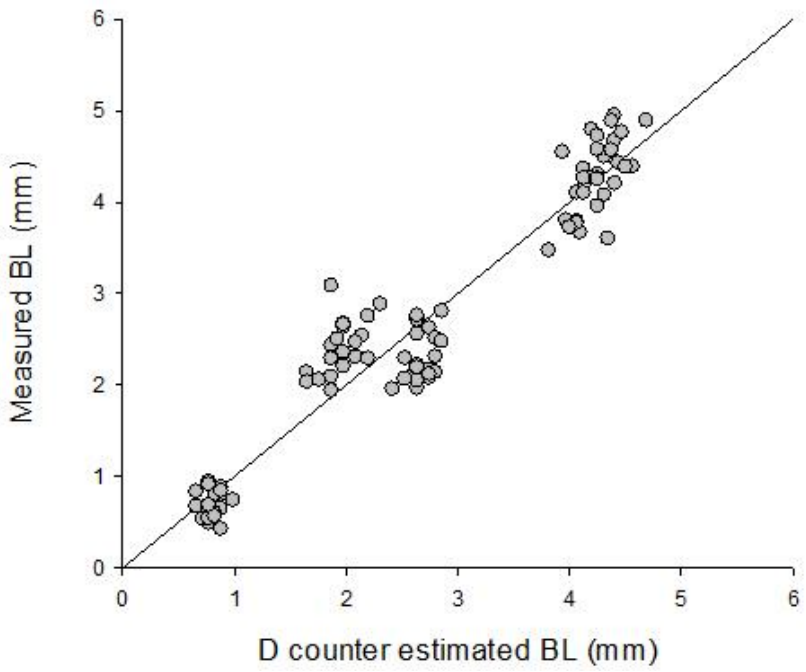


Figure S4. 2- Matching between D counter values and measured BL.

Regarding the regression of both measurement techniques (figure S4.2), it is defined by the following equation: Length-stereomicroscope = 0,2098 + 0,9210 x D counter. There is a good agreement between the values of manual measurement and D counter, as given by the fitting parameters of the regression: r^2= 0.9211, n= 93, p=<0.0001.

3. Matching between measured the algae absorbance given by the spectrophotometer and the D counter

The comparison between the algae absorbance (ABS) given by the spectrophotometer at 440 nm and the D counter are depicted in figure S4.3.

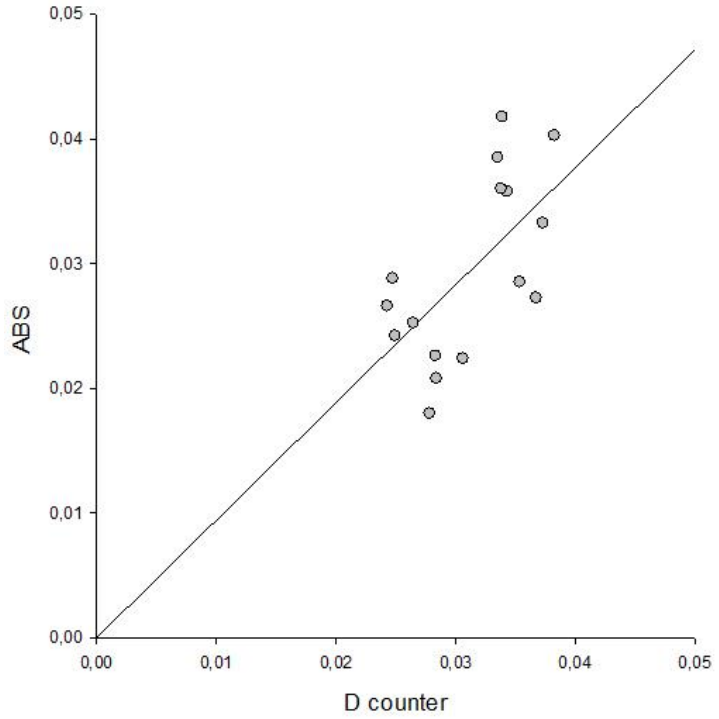
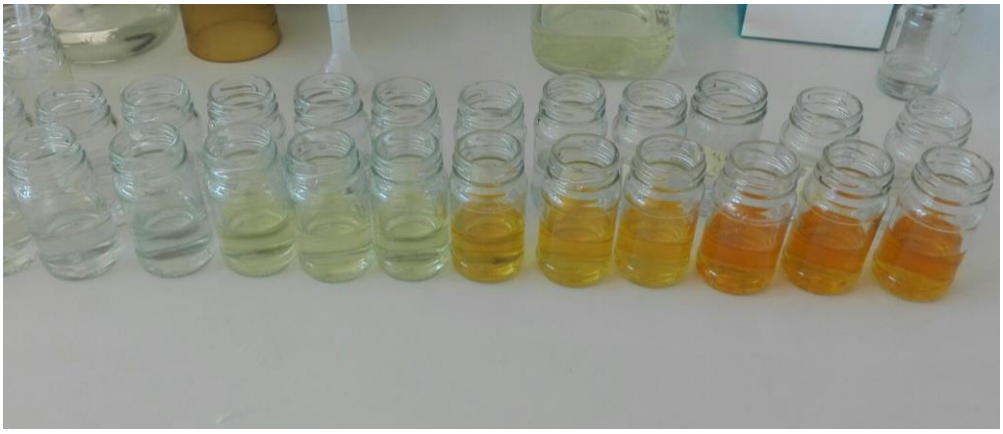


Figure S4.3- Matching between ABS at spectrophotometer and D counter values.

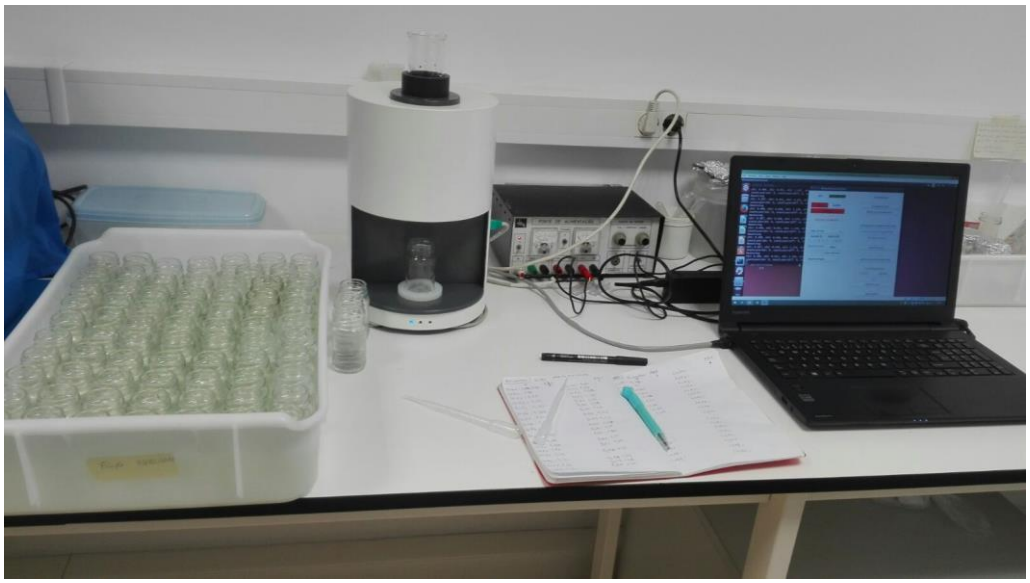
Regarding the regression of both measurement techniques (figure S4.3), it is defined by the following equation: spectrophotometer = 1,0766E-017 x D counter. There is a reasonable match between absorbance given by the spectrophotometer and D counter, as given by the fitting parameters of the regression: r^2= 0.4099, n= 16, p= 0.0076.

Annex 1: Photo of measurement of ammonia

(Different vials, with different colors proportional to the amount of ammonia present in each vial)



Annex 2: Photo of workplace with the equipment (D counter)



Annex 3: Print screen of the D counter software